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(71) Applicant *(for all designated States except US)*: **PERICOR SCIENCE, INC.** [US/US]; One Kendall Square, Building 600, PMB 299, Cambridge, MA 02139 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **GREEN, Howard** [US/US]; 82 Williston Street, Brookline, MA 02146 (US). **RANDO, Robert, R.** [US/US]; 60 Montvale Road, Newton Center, MA 02459 (US).

(74) Agent: **GATES, Edward, R.**; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

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(54) Title: LYSINE OXIDASE LINKAGE OF AGENTS TO TISSUE

(57) Abstract: Methods, products and kits are provided for attaching agents to body tissues using lysine oxidase.

LYSINE OXIDASE LINKAGE OF AGENTS TO TISSUE

Field of The Invention

This invention relates to the linkage of agents to tissue by lysine oxidase and involves methods, products and kits relating thereto.

Background of The Invention

Transglutaminases are a family of calcium-dependent enzymes mediating covalent crosslinking reactions between specific peptide bound γ-glutamyl residues and various primary amino groups of peptide-bound lysines or polyamines, acting as amine donor substrates (Davies, et al., *Adv. Exp. Med. Biol.* 250, 391-401, 1988). These enzymes stabilize biological structures via the formation of isopeptide crosslinks. In mammals, at least five enzymatically active transglutaminases have been identified, cloned and sequenced. The number of proteins acting as glutaminyl substrates for transglutaminases is restricted, and no obvious consensus sequence around these substrates' glutamines has been found.

More recently, people in the art determined that as long as polypeptides including stretches of polyglutamine are rendered sufficiently soluble by the flanking residues, all were excellent substrates of transglutaminase. It also is described in U.S. Patent 5,525,336 (the disclosure of which is incorporated herein by reference in its entirety) that transglutaminases and corneocyte proteins, the natural substrates of transglutaminases, can be used together as cosmetic treatments to crosslink preparations of corneocyte proteins to the outer layer of skin, hair or nails to form a protective layer on the skin, hair or nails.

U.S. Patent 5,490,980 describes selecting agents having or modifying agents to have an aliphatic amine, and then attaching those agents to skin, hair or nails using transglutaminase. While the idea was sound in principle, in practice the '980 applicants achieved results that were barely above background. (See Example Section of '980 patent). An aliphatic amine was applied in the examples as a single linking molecule or prophetically in clusters (according to a formula in the '980 patent).

Lysine oxidase (EC 1.4.3.14) catalyzes the oxidative transformation of the ε-amino group of lysine to an aldehyde group. The resultant aldehyde group in turn undergoes an intermolecular Schiff base formation with amino groups of proteins or an aldol condensation with other aldehyde groups to forge cross links. Lysine oxidase (CAS # 70132-14-8;) is commercially available from Sigma Chemical Co., St. Louis, MO) (Cat. # L6150), and United States Biochemical Company (Cat. # 18612).

Summary of The Invention

It has been discovered that lysine oxidase (e.g., lysines) can be used to attach agents (including microparticles containing agents) to proteinaceous material such as body tissue. Lysine oxidase reacts with lysine amines to form aldehydes (i.e., lysyl aldehydes). As used herein, the lysyl aldehydes formed from lysyl amines by lysine oxidase are referred to as "lysine oxidase products" or "products of lysine oxidase." It also has been discovered that molecules, including native peptides and conjugates according to the invention, can be screened to determine those that can be substrates of lysine oxidases and those that can react with products of lysine oxidase, and then such molecules can be attached to a body tissue. Methods of attaching agents to body tissue and methods of screening molecules that are useful in such a process using lysine oxidase are provided. In addition, compositions of matter suitable as substrates for lysine oxidase, or which can react with lysine oxidase products such as aldehydes, and kits containing such molecules together with lysine oxidase are also provided.

According to one aspect of the invention, a method is provided for attaching an agent to a body tissue. An agent attached to a linker that is selected from the group consisting of compounds that are substrates of lysine oxidase (i.e., it possesses a lysine residue) and compounds that react with lysine oxidase products (i.e., it possesses an aldehyde or amine),k and the conjugate of the agent and linker is applied to the body tissue. In one embodiment, lysine oxidase also is applied to the body tissue, in an amount effective to permit crosslinking the agent which is attached to the linker to the body tissue. Thus attachment of the agent to the tissue occurs via the linker. Crosslinking then is allowed to occur. As used herein, an effective amount of lysine oxidase can be defined in terms of production of lysine oxidase products (e.g., aldehydes from lysine amines) or in terms of crosslinking of such products to their counterpart reactive molecules (e.g., amines and other aldehydes). In one embodiment, the lysine oxidase is applied to the body tissue first.

Thus, in some embodiments, the linker comprises a lysine oxidase substrate preferably in the form of a lysine residue. In these embodiments, the lysine oxidase acts on the linker and the linker can then react with an amine in the tissue. The lysine oxidase also could act on the body tissue and the linker to generate aldehydes on both. These aldehydes are then able to react spontaneously with each other.

In other embodiments, the linker comprises a reactive moiety that reacts with lysine oxidase products (i.e., it reacts with aldehydes). Such moieties are preferably amines or aldehydes. A lysyl aldehyde spontaneously reacts with an amine to form a Schiff base, or with an aldehyde to form an aldol (in an aldol condensation reaction).

Thus, the linker may comprise a molecule selected from the group consisting of at least one amine, aldehyde or lysine, at least two contiguous linked amines, aldehydes or lysines, at least three contiguous linked amines, aldehydes or lysines, at least four contiguous linked amines, aldehydes or lysines, or at least five contiguous linked amines, aldehydes or lysines. In preferred embodiments, the linker comprises 4 or more contiguous amines, aldehydes or lysines attached directly to one another by covalent bonds, such as peptide bonds.

In certain embodiments, the linker comprises a polymer. The polymer may be a polymer of amino acids. In some embodiments, at least 20%, at least 30%, or at least 40% or more of the amino acids are lysines.

In certain embodiments, the method further comprises first treating the body tissue to expose reactive molecules in the tissue (such as lysines).

In other embodiments, the method further comprises first attaching to the body tissue a complementary linker, and attaching the complementary linker and the agent to one another by crosslinking the linker (which is attached to the agent) and the complementary linker by the lysine oxidase. In some embodiments, the crosslinking occurs after exposure to lysine oxidase. In some embodiments, the complementary linker is attached to the body tissue by applying to the body tissue the complementary linker, applying to the body tissue an amount of lysine oxidase effective for crosslinking the complementary linker to the body tissue, and allowing said crosslinking to occur. In certain embodiments, the linker or complementary linker comprise a polymer rich in lysine. In preferred embodiments, the polymer rich in lysine has 4 or more contiguous lysines directly attached to one another by peptide bonds. The complementary linker may be crosslinked to the body tissue using transglutaminase, in some embodiments.

In any of the foregoing embodiments, the agent itself may, or may not, be a substrate of lysine oxidase. Alternatively, the agent may be, or may not be, capable of spontaneously reacting with a lysyl aldehyde. In another embodiment, the agent does not itself react with lysine oxidase substrates. In any of the foregoing embodiments, the body tissue may be

integument, skin, hair, nails, a wound bed, and/or internal body tissue. When in any of the foregoing embodiments, the body tissue is skin, hair, and/or nails, the agent may be a cosmetic, a bulking agent, a sunscreen agent, and/or a coloring agent. In some embodiments, the agent may be an enzyme. In certain embodiments, the enzyme includes a cholinesterase and/or a phosphodiesterase. In important embodiments, the agent is an anti-nerve agent. In related embodiments, the agent is an enzyme which degrades nerve agents and may be selected from the group consisting of OPAA anhydrolase and squid type OPA anhydrase. In any of the foregoing embodiments, the agent may be a pharmaceutical agent, a ligand of a ligand-receptor complex, and/or a receptor of a ligand-receptor complex. In certain embodiments, the bond between the agent and the linker is a hydrolyzable bond. In some embodiments, the agent may be a nonprotein. In some embodiments, the agent and/or linker are provided as a microparticle. In other embodiments, the agent and/or linker are not provided as part of a microparticle.

In one aspect, the invention provides a method for attaching an agent to a body tissue comprising first attaching to the body tissue a linker which is covalently bondable to the agent in the presence of lysine oxidase, then applying to the body tissue having the linker attached thereto an agent which is covalently bonded to the linker in the presence of lysine oxidase, applying to the body tissue lysine oxidase in an amount effective to crosslink that agent to the linker and allowing the crosslinking to occur. In one embodiment, the linker is a substrate of lysine oxidase and the linker is attached to the body tissue by applying to the body tissue the linker, applying to the body tissue the lysine oxidase in an amount effective to crosslink the linker to the body tissue and allowing the crosslinking to occur. In one embodiment, the a polymer rich in lysine is the linker. In another embodiment, the agent comprises a polymer rich in lysine. In one embodiment the agent does not comprise a microparticle, and in another the linker does not comprise a microparticle. The agent may be any of the agents described herein including an enzyme that degrades nerve agents such as OPPAA anhydrolase or squid type OPA anhydrase.

According to another aspect of the invention, a method is provided for attaching an agent to a body tissue. The method involves selecting an agent that is a substrate for lysine oxidase or an agent that can react (spontaneously) with a lysine oxidase product (i.e., a lysyl aldehyde). The agent, in an isolated form, then is applied to the body tissue in the presence of a sufficient amount of lysine oxidase to crosslink the isolated agent to the body tissue. As

mentioned earlier, this amount embraces the amount sufficient for converting lysyl amines (lysine oxidase substrates) to lysyl aldehydes (lysine oxidase products). Crosslinking then is allowed to occur. The crosslinking in all embodiments of the invention may occur in the absence or the presence of lysine oxidase, provided a sufficient number of lysine oxidase products have been produced prior to the crosslinking. In one embodiment, the agent can be attached to a linker, and the linker may not be native to the agent. It also is the case that the agent can be a native agent free of attachment with linkers (or molecules) not native to the agent. In some embodiments, the agent and/or linker are provided as a microparticle. In other embodiments, the agent and/or linker are not provided as part of a microparticle.

In any of the foregoing embodiments, the linker can be any number of a variety of linkers. In some embodiments, the linker is at least one amine or aldehyde. The linker, likewise, may comprise two or more contiguous linked amines or aldehydes. In a preferred embodiment, the linker is a polymer. The polymer may be rich units having amines or aldehydes. A polymer rich in amines or aldehydes is a polymer with at least 20% of units having amines or aldehydes or it is a polymer having at least three, preferably four and most preferably five contiguous, linked units comprising amines or aldehydes, preferably linked by peptide bonds. The polymer rich in units having amines or aldehydes can be a polymer that contains at least 30%, at least 40%, or even 50% or more of such units. In any of the foregoing embodiments (and other embodiments of the present invention), but particularly where the polymer is rich in both glutamines and lysines, a transglutaminase may also be used as well as the lysine oxidase according to the invention.

In certain aspects of the invention, lysine groups are first prepared for crosslinking through exposure to lysine oxidase which converts amines to aldehydes. In certain preferred embodiments, the methods described above involve first preparing the body tissue for the attachment of the agent to the body tissue. In one important embodiment, a complementary linker that is attachable to the linker by lysine oxidase or otherwise is first attached to the body tissue to provide multiple, accessible linking sites for the attachment of the linker to the body tissue. The complementary linker can be attached to the body tissue by any suitable means, but preferably is attached by applying the complementary linker to the body tissue, and applying lysine oxidase or transglutaminase to the body tissue in an amount effective for crosslinking the complementary linker to the body tissue. Crosslinking then is allowed to

occur. Preferably, the complementary linker is a polymer rich in lysine, or both glutamine and lysine.

As used herein, attachment or crosslinking by lysine oxidase embraces the reaction of lysines with lysine oxidase (to form aldehydes) followed by the spontaneous reaction of so generated aldehydes with other aldehydes (i.e., an aldol condensation reaction) or with amine groups such as those of lysine (i.e., a Schiff base formation) to form crosslinks.

Layers of such complementary linkers can be attached to body tissue. To exemplify, polylysine could first be attached to the surface of a body tissue using lysine oxidase. Subsequently polylysine could be attached to the polylysine by lysine oxidase, and so forth, to create layers of such molecules in the body tissues, for example, for bulking purposes or to provide an even, continuous bed of reactive groups for linking an active agent to the body tissue. Transglutaminase could be used for this purpose as well. As used herein a pair of molecules which are covalently joined are said to be "complementary" molecules.

As will be understood, multiple layers of polymers may be attached to the body surface for priming the body surface for attachment of an agent. For example, polymers comprising polylysine may first be attached to a body tissue. Then, agents attached to polylysine may be applied to the coated body surface and easily attached to the exposed lysines of the polylysines on the body surface. Similarly, agents attached to linkers containing aldehyde groups, or agents which themselves have aldehyde groups, may be applied to the coated body surface and easily attached to the exposed lysines of the polylysines on the body surface. Optionally, the exposed lysines may be treated with lysine oxidase before, during or after exposure to the agents. Preferably, the body surface or the linker is pretreated with lysine oxidase.

In some embodiments, the agent (or native agent) is not itself a substrate of lysine oxidase nor can it react with lysine oxidase products (i.e., it does not contain lysine or amines or aldehydes). Thus, it is required that the agent be attached to a compound that is a substrate of lysine oxidase or to a molecule that can spontaneously react with lysine oxidase products whereby the agent may be attached to the body tissue by such compounds which provide the linker. The compound may comprise aliphatic amines which can be oxidized by lysine oxidase and then coupled to amines or aldehydes, or alternatively, it may itself contain amines and aldehydes and thus can couple to aldehydes generated by the action of lysine oxidase. It

also is possible to modify peptide agents by adding a side group, whereby the agent which itself is not a substrate of lysine oxidase is converted to a substrate of lysine oxidase.

According to the foregoing methods, the agents, and agents attached to linkers, are attached to proteinaceous material. The preferred proteinaceous material is body tissue, including the integument, a wound bed, internal organs or internal tissue. Even more preferred in some embodiments are the skin, nails and hair.

According to the foregoing methods, the agent can be any variety of agents, including cosmetics such as bulking agents, coloring agents, sunscreen agents, hair conditioning agents, hair fixative agents, anti-foaming agents, moisturizing agents, including humectants, and depilatories (i.e., hair removal agents), vitamins, film forming agents such as those used in hair fixatives or wound healing, insect repellants including louse repellents, anti-nerve gas or anti-neurotoxin agents such as enzymes including cholinesterase and phosphodiesterase, pharmaceutical agents, ligands of ligand-receptor complexes, receptors of ligand-receptor complexes, and the like. In some embodiments, the agent is an enzyme that degrades nerve agents and may be selected from the group consisting of OPAA anhydrolase and squid type OPA anhydrase. In one embodiment, the agent is a member of a noncovalent coupling pair, such as biotin and avidin, to provide a universal linker as discussed in greater detail below. In certain embodiments, particularly those employing pharmaceutical agents, the bond between the agent and the linking molecule can be a bond which cleaves under normal physiological conditions or which can be caused to cleave specifically, for example, by exposure to light. In many instances where the agent is not itself a substrate of lysine oxidase, the agent is a non-protein.

According to another aspect of the invention, a method is provided for attaching an agent to a body tissue. A linker which is covalently bondable to the agent by any means including the use of lysine oxidase is attached to the body tissue. Then, an agent is applied to the body tissue. Lysine oxidase also is applied to the body tissue, in an amount effective to crosslink the agent to the linking molecule. Crosslinking then is allowed to occur. The linker can be attached to the body tissue by any suitable means, but in one embodiment the linker is a substrate of lysine oxidase or transglutaminase and preferably it is attached to the body tissue by applying the linker to the body tissue together with lysine oxidase or transglutaminase, the lysine oxidase or transglutaminase being present in an amount effective to crosslink the linker to the body tissue. Preferred linkers are lysine and polymers of

glutamine and/or lysine. Most preferred are polymers that are rich in lysine, or both glutamine and lysine.

In any embodiment, the agent can be any substance including those listed above but also including labels, extracellular matrix proteins and corneocyte proteins. Preferred body tissues are as described above. In some embodiments, the agent, linker or conjugate does not comprise a microparticle.

According to another aspect of the invention, a method is provided for attaching an agent to a body tissue. The method involves first attaching to the body tissue a linker which is covalently bondable to the agent in the presence of lysine oxidase. Then, the method involves applying to the body tissue having the linker attached thereto an agent that is covalently bonded to the linker, in the presence of the sufficient amount of lysine oxidase effective to crosslink the agent to the linker attached to the body tissue. Crosslinking then is allowed to occur. Preferred agents, linkers and body tissues are as described above.

According to another aspect of the invention, a method is provided for determining whether an agent is a substrate for lysine oxidase or whether it reacts with a lysine oxidase product. The method involves applying the agent in an isolated form to a proteinaceous material such as a body tissue, a body tissue isolate, a polymer rich in amine, such as preferably lysine and the like. Lysine oxidase then is applied to the proteinaceous material in an amount sufficient and under conditions appropriate to crosslink the agent to the proteinaceous material if the agent is a substrate of lysine oxidase or if it reacts with a lysine oxidase product. It then is determined whether the agent covalently binds to the proteinaceous material, covalent binding being indicative that the agent is a substrate of lysine oxidase or if it reacts with a lysine oxidase product. Preferably the agent is an active agent and, in other preferred embodiments, the active agent is a covalent conjugate of a native active agent and a linker not native to the active agent. In other embodiments, the active agent is a native active agent free of conjugation with groups not native to the active agent. Thus, in this aspect of the invention, methods are provided for creating conjugates of active agents and linkers (or linking molecules) and determining whether the conjugates are substrates for lysine oxidase or whether they react with a lysine oxidase product. In certain preferred embodiments active agents such as pharmaceutical agents, cosmetics, sunscreen agents and the like, which are peptides in their native form, are screened to determine whether

they are substrates of lysine oxidase or whether they react with a lysine oxidase product so that they may be attached to body tissue according to the invention.

Alternatively, the proteinaceous material (including lysines) is exposed to lysine oxidase, after which the agent (or linker) is applied to the material. It is then determined whether the agent (or linker) is covalently attached to the material. In yet another variation of this, the agent or linker may be applied to a solid support, lysine oxidase may be applied to the solid support coated with the agent or linker and a detectable label known to have amine, or more preferably, aldehyde groups, is applied to the support. If the detectable label remains attached to the support following washing, then this indicates a covalent bond and that the agent or linker was a substrate for lysine oxidase.

According to another aspect of the invention, a method for attaching an agent to a body tissue is provided. The method involves applying to the body tissue a conjugate of the agent and a linker which is an amine, an aliphatic amine or an aldehyde (preferably the linker being a polymer with at least 3 amines, aliphatic amines or aldehydes spaced along the polymer) applying to the body lysine oxidase in an amount effective for crosslinking the linker to the body tissue, and allowing crosslinking to occur. The amines and aliphatic amines can be the side chain of L or D lysines. D lysines have the advantage of being physiologically more stable than L lysines. Most preferably, the linker may include at least 3, at least 4 and at least 5 contiguous amines, aliphatic amines, lysines or aldehydes attached to one another directly by peptide bonds. The polymer also can be one rich in amines, aliphatic amines, or aldehydes. An example is a polymer rich in lysines, as described above. Preferred agents and body tissues are as described above.

According to another aspect of the invention, compositions of matter are provided. The compositions include conjugates of an agent and a linker, the linker in some embodiments being a substrate of lysine oxidase, in other embodiments being able to react spontaneously with a lysine oxidase product, and in still other embodiments, not being a substrate of transglutaminase. The agent includes a sunscreen agent, a bulking agent, a cosmetic, a hair conditioning agent including an anti-foaming agent or an anti-static agent, a hair fixative agent, a moisturizing agent, including a humectant, and a depilatory agent (i.e., a hair removal agent), a vitamin, a film forming agent such as those used in hair fixatives or wound healing, an enzyme, a coloring agent, a pharmaceutical agent, a member of a ligand/receptor pair, a component of a high-affinity non-covalent coupling pair, a tissue

sealant, an insecticide including louse repellents, an insect repellant, a bactericide, a fungicide, an anti-nerve gas or anti-neurotoxin agent and the like. In one embodiment, the linker is a substrate for lysine oxidase, is not a substrate of transglutaminase, and is not native to the agent. In other embodiments, the linker contains amines or aldehydes and thus can spontaneously react with lysine oxidase treated tissue. In certain embodiments, particularly those involving the pharmaceutical agents, the bond between the agent and the linker or molecule is a hydrolyzable bond. In certain important embodiments, the agent is a non-protein. In other important embodiments, the agent is an active agent. In other important embodiments, the agent, in its native form free of conjugation to the linker, is not itself a substrate of lysine oxidase.

According to other aspects of the invention, kits are provided. One such kit includes a package housing a first container containing an agent attachable to proteinaceous material in the presence of lysine oxidase and a second container containing lysine oxidase. The kit can further comprise a third container housed by the package, the third container containing a linker that is a substrate of lysine oxidase and that is covalently bondable to the agent contained in the first container in the presence of lysine oxidase. The various containers also can contain catalysts, vehicles, calcium, preservatives, buffers, and calcium chelators.

In another aspect, the invention provides a kit comprising a microparticle comprising surface available reactive groups in an amount sufficient to attach the microparticle to a skin surface in the presence of lysine oxidase, and lysine oxidase. The kit further includes in one embodiment, instructions for topically administering the microparticles to a skin surface. In another embodiment, the kit include a complementary linker. The surface available groups may be selected from the group consisting of amines, aldehydes, aliphatic amines, lysine and, in general, substrates of lysine oxidase. Other embodiments that pertain to microparticle compositions as described herein are also embraced.

In yet another aspect, the invention provides a kit comprising a microparticle having surface available reactive groups in an amount sufficient to attach the microparticle to a skin surface in the presence of lysine oxidase, and instructions for topically administering the microparticle to a skin surface, wherein the surface available reactive groups are selected from the groups consisting of aldehydes and amines. In one embodiment, the kit further comprising exogenous lysine oxidase. In one embodiment, the kit further comprises a cleanser. In yet another, it comprises a complementary linker. In yet another embodiment,

the microparticle is provided in a topically administered form selected from the group consisting of an ointment, an aerosol, a gel, and a lotion.

As mentioned above, the tissue can be pretreated to make it more receptive to the action of lysine oxidase. In one embodiment described above, this is accomplished by attaching polymers rich in lysine, or both glutamine and lysine to the body tissue. In other embodiments, the tissue is treated to expose reactive groups by washing, chemical treatment, etc. Detergents and lipases can be used to remove fatty acids and oils. Roughening agents such as pumice, silica and sandpaper can be employed to remove dead tissue and other obstructions, and chemical agents such as sodium hydroxide can be used to expose reactive groups. Combinations of the foregoing are contemplated. The tissue may also be pretreated by exposure to lysine oxidase.

The invention also involves the use of lysine oxidase to 'glue' two tissues together. Two tissues are held in contact with one another in the presence of an effective amount of lysine oxidase, whereby the lysine oxidase causes the crosslinking of the tissue to occur by, for example, converting lysines on both surfaces to reactive aldehydes which can crosslink with each other spontaneously to seal the tissue. The surfaces of the tissues to be glued to one another may be treated with a substrate of lysine oxidase such as polymers rich in lysine to create highly reactive surfaces in the presence of lysine oxidase. These highly reactive surfaces are bonded to one another. In another embodiment, the surfaces of the tissue are first treated with a primary linker to crosslink the primary linker to the surfaces, then a secondary linker complementary to the first is applied to crosslink the primary molecules to one another and glue the tissue. The lysine oxidase may be exogenously supplied. The tissue may be held together by any conventional means, such as sutures, tape, stapes and the like.

The agent also can be in a vehicle such as a microparticle (e.g. a microsphere, a microcapsule, or a nanosphere), the microsphere or microcapsule being rich in lysines, or glutamines and lysines, whereby the microparticle can be attached to a body tissue.

Thus in one aspect, a method is provided of treating a subject to attach microparticles to a body tissue of the subject comprising contacting the body tissue with lysine oxidase in an amount effective to permit crosslinking of the microparticles to the body tissue, contacting the body tissue with microparticles having surface available reactive groups in an amount sufficient to attach the microparticles to the body tissue in the presence of lysine oxidase, and allowing the microparticles to remain in contact with the body tissue for a time sufficient to

permit a layer of microparticles to covalently attach to the body tissue. In important embodiments, the body tissue is an external surface such as a skin surface, nails or hair. Some of the following embodiments recite skin surface as the body tissue, however, it is to be understood that any body tissue can be substituted in these embodiments. In one embodiment, the lysine oxidase is exogenous. In related embodiments, the reactive groups are surface available in an amount sufficient to attach the microparticle to a skin surface in the presence of exogenous lysine oxidase.

In important embodiments, the surface available reactive groups are selected from the group consisting of amines, aldehydes, aliphatic amines or lysines. If the microparticles are to act as substrates of lysine oxidase, then the surface available reactive groups are preferably lysines. If the microparticles are to react with lysine oxidase products, then the surface available reactive groups are preferably amines or aldehydes.

In one embodiment, the layer of microparticles is non-planar. In another embodiment, the microparticles further comprise an agent. The agent may be an active agent, but it is not so limited. In one embodiment, the agent is a non-nucleic acid active agent, while in another embodiment, it is a non-protein active agent. In another embodiment, the agent is a protein agent. In certain embodiments, the active agent is selected from the group consisting of a cosmetic agent, a bulking agent, a hair conditioning agent, a hair fixative, a sunscreen agent, a moisturizing agent, a depilatory agent, an anti-nerve gas agent, a film forming agent, a vitamin, an insect repellant, a coloring agent, a pharmaceutical agent, a ligand-receptor complex and a receptor of a ligand-receptor complex.

In other embodiments, the active agent is not itself a substrate of lysine oxidase. In still other embodiments, the agent is not itself able to react with lysine oxidase products. An agent which is not able to react with lysine oxidase products is defined herein as an agent which lacks both amine and aldehyde groups. In one embodiment, the agent is an enzyme. The enzyme may be an enzyme that degrades nerve agent and may be selected from the group consisting of OPAA anhydrolase and squid type OPA anhydrase.

In one embodiment, the microparticles further comprise a synthetic polymer. The synthetic polymer may be latex or polystyrene, but is not so limited. In another embodiment, the microparticles are porous. In yet another, they are hollow. Depending upon the embodiment, the microparticles have a size selected from the group consisting of greater than 5 μm, less than 5 μm, less than 1 μm, 100 nm to 500 nm, less than 100 nm, 20 nm to 90 nm,

20 nm to 35 nm, less than 20 nm, 1 nm to 10 nm, and 5 nm to 10 nm. These sizes or ranges can be cut offs or can represent average size determinations. The microparticles may be non-biodegradable. In some preferred embodiments, they are water insoluble. In some even more preferred embodiments, they are detergent insoluble. In some embodiments, the microparticles enter the cornified layer of the skin but not the layer of living cells. However, in these latter embodiments, the agent contained within the microparticle may be able to enter the layer of living cells.

In some embodiments, the reactive groups are part of a polymer. The polymer may be covalently attached to the microparticle. In one embodiment, the polymer may be comprised of units at least 20%, at least 30%, at least 40%, or at least 50% of the units carrying reactive groups, wherein the reactive groups are selected from the group consisting of amines, aldehydes, aliphatic amines and lysines. In another embodiment, the polymer is rich in reactive groups at a surface available terminus, anywhere from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15, or more units long, or at a surface available loop. In still other embodiments, the polymer comprises a polymer selected from the group consisting of at least two contiguous linked units each carrying reactive groups, at least three contiguous linked units each carrying reactive groups, at least four contiguous linked units each carrying reactive groups, at least five contiguous linked units each carrying reactive groups, at least ten contiguous linked units each carrying reactive groups, at least fifteen contiguous linked units each carrying reactive groups, and at least twenty contiguous linked units each carrying reactive groups. In some embodiments, the reactive groups in a polymer are the same (i.e., they are all lysines, all amines, all aliphatic amines, or all aldehydes).

The invention provides, in yet other aspects, compositions of microparticles and kits thereof. The foregoing embodiments relating to methods of use of microparticles are equally applicable to the following microparticle composition and kit aspects of the invention. Thus, in one aspect, the invention provides a composition having a microparticle comprising an agent and a polymer rich in amine or aldehyde reactive groups, wherein the amine or aldehyde reactive groups are surface available in an amount sufficient to attach the microparticle to a skin surface in the presence of lysine oxidase. In one embodiment, the lysine oxidase is exogenous, and in another it is endogenous.

In one embodiment, the reactive groups are surface available in an amount sufficient to attach the microparticle to a skin surface in the presence of endogenous lysine oxidase. In another

embodiment, the reactive groups are surface available in an amount sufficient to attach the microparticle to a skin surface in the presence of exogenous lysine oxidase.

In another aspect, the invention provides a composition comprising a microparticle having a non-nucleic acid active agent, and covalently attached surface available reactive groups, wherein the microparticle is 100 nm to 500 nm in size, or other sizes as described herein. In one embodiment, the surface available reactive groups are free pendant groups.

In any of the foregoing embodiments, agents may be derivatized to possess hydrazide reactive groups which react with aldehydes.

These and other aspects of the invention are described in further detail below.

Brief Description of The Drawing

Figure 1 depicts a kit according to the invention.

Detailed Description of the Invention

The invention involves in several aspects the linking of agents to a proteinaceous material. In general, the agents are chemical agents and include, but not are limited to, pharmaceutical agents, enzymes, cosmetics, sunscreen agents, ligands of ligand-receptor pairs, receptors of ligand-receptor pairs, components of high affinity noncovalent bonding pairs, insecticides and repellants, bactericides, fungicides, tissue sealants, labels, structural proteins, chelating agents and the like. In important embodiments, the agent is an anti-nerve gas agent. The agent may be an enzyme that degrades nerve agents and as such may be selected from the group consisting of OPAA anhydrolase and squid type OPA anhydrase. Examples of other agents useful in the invention are listed below.

By active agent it is meant that the agent, once coupled to a biological tissue *in vivo* or *in vitro*, has, maintains or can be released to have a desired activity such as a desired biological activity or therapeutic activity. Examples of active agents are pharmaceutical agents, sunscreen agents, insecticides, bactericides, fungicides, etc.

The agents are linked to proteinaceous material. When used *in vivo*, the agents are attached to a body tissue. Particularly important body tissues as sites of attachment are the integument (including specifically skin, nails, hair, mucous membranes and the surface of the eye), internal organs, internal tissue and wound beds. In *in vitro* applications, the tissue may be a body tissue, a tissue or cell isolate, isolated proteins, synthetic proteins, cell cultures and the like for use, for example, in assay systems according to the invention. In preferred embodiments, the body tissue is skin, nails, and hair.

In certain embodiments, conjugates of agents and linkers (as well as microparticles) are applied, for example, to body tissue and covalently linked to that tissue using lysine oxidase. As described earlier, crosslinking may occur in the presence of lysine oxidase but it need not. Lysine oxidase functions, in important embodiments, to prepare lysines for spontaneous bond formation in subsequent aldol condensation or Schiff base reactions. Thus, lysine oxidase, in some preferred embodiments, is applied to a lysine containing material (such as the body tissue, the linker, the agent or the microparticle (as described below)) and allowed to generate reactive aldehydes from the lysine amine groups. Once these reactive aldehydes are generated (or formed), they can spontaneously react with other substances that contain amines or aldehydes, to form covalent crosslinks.

As used herein, "linking" or "conjugate" means two entities stably bound to one another by any physiochemical means. It is important that the nature of the attachment be of such a nature that it does not impair substantially the effectiveness of the agent or the substrate binding ability of the linker. Keeping these parameters in mind, any linkage known to those of ordinary skill in the art may be employed, covalent or noncovalent. Covalent is preferred. Such means and methods of attachment are well known to those of ordinary skill in the art. An agent attached to a linker according to the invention is therefore a conjugate.

Typically the agents used according to the invention are not themselves, in their native form, substrates for lysine oxidase nor can they spontaneously react with lysine oxidase products. Such agents, however, can be modified according to the invention to render the agent so. This may be accomplished for example by adding an amine or an aldehyde side group(s) to an appropriate peptide moiety of the agent (i.e., a "modified" agent) or by covalently coupling an amine or aldehyde containing substance (such as lysine or polylysine) to the agent to form a conjugate that is useful. The most preferred method is to couple polylysine, to the agent to form an appropriate conjugate. Such a conjugate could function as a substrate for lysine oxidase and, as well, it could spontaneously react with a lysine oxidase product. In particular embodiments, the agent is preferably modified to contain aldehydes or it is conjugated to an aldehyde containing substance.

In some embodiments, the most preferred linkers (or linking molecules) are polymers rich in lysine. A polymer rich in lysine is a molecule wherein at least 20% of the units of the polymer are lysine, or wherein the molecule includes at least 3, preferably 4 and most preferably 5 contiguous, linked lysines. It should be understood, however, that as few as one